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Protozoan and bacterial pathogens in tick salivary glands in wild and domestic animal environments in South Africa

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ABSTRACT

A total of 7364 ticks belonging to 13 species was collected from 64 game animals (belonging to 11 species) and from 64 livestock animals (cattle and sheep) living in close vicinity at 6 localities in 3 South African Provinces (Free State, Mpumalanga, and Limpopo). The geographic distribution of all tick species was congruent with the literature except for *Haemaphysalis silacea*. From each infested host, a maximum of 10 males and 10 females of each tick species were dissected to isolate the salivary glands. Salivary glands were screened for tick-borne pathogens using polymerase chain reaction followed by reverse line blotting and sequencing. This approach allowed us to evaluate the exposure of wild and domestic hosts to tick-borne pathogens in their respective environments. Among the 2117 examined ticks, 329 (15.5%), belonging to 8 species, were infected and harboured 397 infections. Among those, 57.7% were identified to species level and were assigned to 23 pathogen species of the genera *Babesia*, *Theileria*, *Anaplasma*, and *Ehrlichia*. In 3 out of 6 localities, salivary glands from ticks infesting wild ruminants displayed significantly higher infection prevalence and pathogen mean density than salivary glands from ticks infesting livestock animals. Four piroplasm species [*Theileria bicornis*, *Babesia* sp. (sable), *Theileria* sp. (giraffe), and *Theileria* sp. (kudu)] were detected for the first time in ticks. The tick species *Rhipicephalus evertsi evertsi*, *Rhipicephalus (Boophilus) decoloratus*, *Hyalomma rufipes*, *Rhipicephalus appendiculatus*, and *Amblyomma hebraeum* were associated with a broader pathogen range than previously known, and thus new vector–pathogen combinations are described. In addition, previously unknown coinfection patterns in tick salivary glands are reported.

Introduction

Ticks are vectors of a great variety of tick-borne pathogens. In the recent decades, the development of molecular tools increased their known number and their known variety. The majority of tick-borne pathogen species that circulate between ticks and both game and livestock animals have been reported in the vertebrate hosts they infect and less in the ticks. Indeed, in early studies that aimed to detect tick-borne pathogens in wild African ungulates, animal sera were screened (Neitz and Du Toit, 1932; Neitz, 1935; Löhr and Meyer, 1973; Löhr et al., 1974; Carmichael and Hobday, 1975). More recently, in studies reporting tick-borne pathogens affecting game animals, host blood was examined, but not the associated tick species (Nijhof et al., 2003, 2005; Spitalska et al., 2005; Brothers et al., 2011; Oosthuizen et al., 2008, 2009; Pfitzer et al., 2011). In addition, several tick-borne pathogens with known tick vectors display a broader host range than formerly thought. An example is *Theileria* sp. (sable) that infects the sable (*Hippotragus niger*) and roan (*H. equinus*) antelope and that has been newly described in red hartebeest (*Alcelaphus buselaphus*) (Spitalska et al., 2005), cattle (Yusufmia et al., 2010), and nyala (*Tragelaphus angassii*) (Pfitzer et al., 2011). For these pathogens, a broader host range could mean that additional, so far unknown vectors, especially when associated with a wide host variety, might be involved in their transmission. From this point of view, the two-host tick *R. e. evertsi*, the three-host ticks *Amblyomma hebraeum* and *R. appendiculatus* as well as the one-host tick *R. (Boophilus) decoloratus* are good candidates. These tick species were recorded from a great variety of host species and are geographically widely distributed in southern Africa (Walker et al., 2000, 2003).

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Therefore, in this study, we focused on the presence of *Babesia*, *Theileria*, *Anaplasma*, and *Ehrlichia* in the salivary glands of different tick species collected from wild and domestic ruminants in South Africa. Detection of pathogens in salivary glands of ticks allowed us to distinguish pathogens that infected the ticks before they attached to the hosts from those taken up by ticks during their blood meal. Thus, this approach allows to evaluate the exposure of wild and domestic ruminants to tick-borne pathogens in their respective environments. The presence of a pathogen in tick salivary glands strongly suggests that it may be transmitted to the host, but this is not sufficient to prove its vector role, which would require transmission experiments. The purpose here was (i) to evaluate the possible role of ticks in the transmission of recently described pathogen species, (ii) to verify whether pathogens with a broad host range have a broader vector range than currently known, (iii) to investigate the exposure of game and livestock to tick-borne pathogens in their respective environments, and (iv) to obtain information on coinfections in ticks.

Materials and methods

Study areas

Ticks were collected in 2009 (May to July), 2010 (January to May), and 2011 (April to June) at 6 localities in 3 South African Provinces. In the Free State, 3 provincial nature reserves, Tüssen-Die-Riviere, Willem Pretorius, and Sandveld, as well as several livestock farms at their surroundings were investigated (highveld). In the Mpumalanga Province, one game farm and one livestock farm were investigated near Bethal (highveld). Finally, 5 game farms and 5 livestock farms were investigated in the Limpopo Province in the Thabazimbi and Lephalale areas (lowveld).

Tick sampling

At each locality, tick samplings were performed on domestic and wild animals within the same week. Ticks were sampled from an equal number of livestock animals living on farms sharing a common border with the reserves or game farms or located in the close surroundings (within the range of 40 km). Ticks were collected from game animals during game capture, culling operations, and hunts. Cattle were maintained in holding facilities during sampling, and sheep were immobilised by hand in small camps. Hosts were visually examined for ticks; palpation helped to localise specimens attached on flanks, back, belly, neck, and legs. All the found ticks were removed with tweezers. Tick identification was performed according to Matthysse and Colbo (1987) and Walker et al. (2000, 2003). Ticks were pooled per species, host, and developmental stage and stocked in alcohol in 50-ml labelled tubes.

Detection and identification of pathogen species in tick salivary glands

From each individual host, a maximum of 10 males and 10 females from each tick species was analysed. To distinguish pathogens that had infected ticks before they attached to the host from those taken up by ticks during their current blood meal, each tick was dissected and the salivary glands of dissected ticks were analysed for pathogens. Salivary glands were carefully removed with tweezers – special attention was paid to avoid contamination with the midgut, and washed twice in PBS in 96-well plates (Milian®, Geneva, Switzerland). Instruments were sterilised for a few seconds in 5 M HCl and 5 M NaOH (Aktas et al., 2009) and dried with sterile wipes between each dissection.

DNA from tick salivary glands was extracted using QIAamp® DNA Micro kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer's instructions with modifications. Tissue lysis buffer and proteinase K were added in the 1.5-ml tubes before the salivary glands. DNA was stored at −20 °C.

An approximately 500-base pair (bp) fragment of the 16S ribosomal RNA (rRNA) gene spanning the hypervariable V1 region of the genera *Anaplasma* and *Ehrlichia* and an approximately 400-bp fragment of the 18S rRNA gene spanning the V4 hypervariable region of the genera *Babesia* and *Theileria* were amplified by PCR (Tonetti et al., 2009). Positive control, included in each run, consisted of DNA of *A. phagocytophilum* (provided by Ana Sofia Santos, Instituto Nacional de Saude, Lisboa, Portugal) and *B. divergens* (provided by Simona Casati, Institut Cantonal de Microbiologie, Bellinzone, Switzerland). PCR products were analysed using reverse line blotting (RLB) (Tonetti et al., 2009). In addition to the original 15 oligonucleotide probes, which are listed in Tonetti et al. (2009) (2 genus-specific Babesia/Theileria and Anaplasma/Ehrlichia, and 13 species-specific), 26 probes were added: one genus-specific Theileria spp. (Nagore et al., 2004) and 25 species-specific probes, B. ovis, B. crassa (Schnittger et al., 2004), B. major (Georges et al., 2001), Babesia sp. (sable) (Oosthuizen et al., 2008), B. caballi (Butler et al., 2008), B. occultans (Ros-Garcia et al., 2011), B. orientalis (Hea et al., 2011), B. gibsoni (from Pfitzer, 2009), B. rossi (Matjila et al., 2004), B. bicornis (Nijhof et al., 2003), B. motasi, Theileria sp. (greater kudu), Theileria sp. (sable) (Nijhof et al., 2005), T. separata, T. lestoquardi, T. ovis (Schnittger et al., 2004), T. buffeli (Gubbels et al., 1999), T. bicornis (Nijhof et al., 2003), Theileria sp. (buffalo) (Oura et al., 2004), T. equi (Butler et al., 2008), T. annulata (Georges et al., 2001), Ehrlichia sp. (Omatjenne) (Bekker et al., 2002), and 4 A. phagocytophilum (from Pfitzer, 2009) that replaced the original degenerated probe. Samples reacting only with the Babesia/Theileria probe were considered *Babesia* spp. since a genus-specific probe was included for the genus *Theileria*. To test for theoretical specificity, oligonucleotide probes were aligned with various sequences of targeted species available from the National Centre for Biotechnology Information (NCBI) using a software package: CLC Sequence Viewer 6 (CLC bio, Aarhus, Denmark).

Sequencing

PCR products that reacted only with genus-specific probes Babesia/Theileria, Theileria spp., or Anaplasma/Ehrlichia and did not hybridise with the panel of species-specific probes were sequenced. Prior sequencing, PCR products were purified using Wizard® SV and PCR Clean-Up System (Promega, Madison, USA) following the manufacturer's instructions except that we eluted with 35 μl rather than 70 μl Nuclease-Free Water. Sequencing was performed by Microsynth AG (Balgach, Switzerland). Sequences were compared and corrected with CLC Sequence Viewer 6 (CLC bio, Aarhus, Denmark) and Bioedit (Tom Hall Ibis Biosciences, Carlsbad). Corrected sequences were compared with available sequences retrieved from GenBank using NCBI Basic Local Alignment Search Tool (BLAST).

Data analysis

Data were analysed with "R" 2.14 for Windows (R Development Core Team, 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: http://www.R-project.org/), using software packages (Skaug et al., 2010; Husson et al., 2012). A generalised linear mixed model (GLMM) with negative binominal errors was used to evaluate tick salivary gland infection (taking into account the 4 main infected tick species) collected from wild and domestic ruminants originating from 6 localities. In each locality, the significance of the factors LOCATION and HOST TYPE (i.e. ticks infesting wild vs. domestic ruminants) as well as the

LOCATION–HOST TYPE interaction was assessed, *P* values were considered significant when below 0.05. In order to compute whether associations between pathogen species in coinfections were significant a permutation test proposed by Raup and Crick (1979), adapted to R by Clua et al. (2010) constrained to locations was used. Here, *P* values were considered significant when below 0.0001 (Bonferroni corrected).

Results

Tick sampling

A total of 7364 ticks belonging to 13 species was collected from 13 ruminant species (Table 1). Five tick species contributed 96.5% of the total sampling: *R. (B.) decoloratus* ($n = 2611$), *R. e. evertsi* ($n = 2225$), *Margaropus winthemi* ($n = 1085$), *A. hebraeum* ($n = 767$), and *Hyalomma rufipes* ($n = 417$), and 71% of them were collected from wild animals (Table 1). All tick species were detected in areas that were congruent with their known geographic distribution except *Haemaphysalis silacea*, which was recorded at Willem Pretorius game reserve (central Free State) (Supplementary Table S1). The 2 most abundant tick species were collected at each of the 6 sampling sites, *R. (B.) decoloratus* infested 9 ruminant species and *R. e. evertsi* all host species.

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2013.10.003.

Infection of tick salivary glands

Among the 2117 examined ticks, 329 (15.5%) were infected, and they harboured 397 infections (Table 2). The majority (95%) of the infections were observed in 4 tick species: *R. e. evertsi* harboured 48.6% of the infections (193/397), *R. (B.) decoloratus* 24.7% (98/397), *A. hebraeum* 11.8% (47/397), and *Hy. rufipes* 9.6% (38/397) (Table 2). Five tick species (*Ixodes rubicundus*, *M. winthemi*, *Hae. silacea*, *Otobius megnini*, and *R. zambeziensis*) were free of pathogens. One hundred sixty-eight infections were identified at genus level only: 69 *Babesia* spp., 56 *Theileria* spp., and 43 *Anaplasma* or *Ehrlichia* spp. Sequencing of all these samples did not allow further identification. The remaining infections ($n = 229$) (57.7%) were identified to species level and were assigned to 23 pathogen species, all known to occur in South Africa, except *T. annulata* (Table 2). Among them were 46 identified by sequencing (Table 3). *Theileria* sp. (sable) ($n = 38$; 9.6%), *T. buffeli* ($n = 37$; 9.3%), *T. bicornis* ($n = 27$; 6.8%), *Ehrlichia* sp. (EU191229.1) ($n = 23$; 5.8%), and *T. separata* ($n = 18$; 4.5%) were the most frequent (Table 2).

Pathogen distribution among tick species

Nine pathogen species were detected in one tick species only. *Theileria equi*, *T. separata*, *A. ovis*, and *A. platys* were detected in *R. e. evertsi*; *B. occultans* and *Babesia* sp. (sable) were only detected in *Hy. rufipes*, and *Theileria* sp. (kudu), *Anaplasma centrale*, and *Ehrlichia ruminantium* in *R. appendiculatus*, *R. gertrudae*, and *A. hebraeum*, respectively (Table 2). Seven pathogens were detected in 2 tick species. *Babesia bigemina*, *B. caballi*, *A. bovis*, *T. ovis*, and *Theileria* sp. (giraffe) were detected in *R. (B.) decoloratus* and *R. e. evertsi*. *Theileria bicornis* was detected in *R. e. evertsi* and *R. appendiculatus*. Finally, *T. mutans* was detected in *R. e. evertsi* and *A. hebraeum*. Four pathogen species were identified in 3 tick species. *Theileria* sp. (sable) was detected in *R. e. evertsi*, *R. (B.) decoloratus*, and *A. hebraeum*. *Theileria taurotragi* was detected in *R. appendiculatus*, *R. e. evertsi*, and *Hy. rufipes*. *Anaplasma marginale* was detected in *R. gertrudae*, *R. (B.) decoloratus*, and *R. e. evertsi*. *Ehrlichia* sp. (EU191229.1) was detected in *R. (B.) decoloratus*, *R. e. evertsi*, and *Hy. rufipes*. Finally, *T. buffeli* and *E. ovina* were detected in 4 tick species: *Theileria buffeli* was detected in *R. (B.) decoloratus*, *R. e. evertsi*, *Hy. rufipes*, and *R. appendiculatus*, while *E. ovina* was detected in *R. (B.) decoloratus*, *R. e. evertsi*, *Hy. rufipes*, and *R. gertrudae*.

Salivary gland infection of ticks attached to wild versus domestic ruminants

Ticks that were attached to wild ruminants showed an infection prevalence of 23.3% (227/973) and harboured 284 infections, while ticks attached to domestic ruminants displayed an infection prevalence of 11.5% (102/885) and harboured 113 infections (Tables 4a and 4b). The differences were mainly due to *Theileria* spp. (16.1% vs. 8.4%), *Theileria* sp. (sable) (12.6% vs. 1.7%), *T. buffeli* (10.2% vs. 6.7%), *T. bicornis* (7.4% vs. 5%), and *T. separata* (4.9% vs. 3.4%) (Tables 4a and 4b).

Taking into account the 4 main infected tick species [*R. (B.) decoloratus*, *R. e. evertsi*, *A. hebraeum*, and *Hy. rufipes*], salivary gland infection (prevalence of infection and mean density of pathogens) varied significantly among the sampling localities (Table 5). The infection pattern was impacted by the factors LOCATION and HOST TYPE as well as by the interaction of both factors (Glmm analyses $P < 0.001$ for the factors LOCATION, HOST TYPE,and their interactions). In contrast,the intensities of infection did not significantly differ among host types and/or locations at the 5% risk (data not shown). In Sandveld, Thabazimbi, and Lephalale, infection prevalence and mean density of pathogens were significantly higher in salivary glands of ticks infesting wild than domestic hosts. Similarly, the tick mean density was significantly higher on wild animals than on domestic animals in Sandveld and Thabazimbi (data not shown).

Coinfections

Among infections identified at species level, 93 (44.7%) were coinfections: 57 (61.3%) involved 2 pathogens, 24 (25.8%) 3, and 12 (12.9%) 4 (data not shown). *Rhipicephalus e. evertsi* showed the highest prevalence of coinfections ($n = 67$; 72%), followed by *R. (B.) decoloratus* ($n = 21$; 22.6%), *R. appendiculatus* ($n = 3$; 3.2%), and *Hy. rufipes* ($n = 2$; 2.2%) (data not shown). Six associations involving 5 pathogen species were significant: *Theileria* sp. (sable) with *T. separata*; *Theileria* sp. (sable) with *T. bicornis*; *T. separata* with *T. bicornis*; *T. separata* with *T. buffeli*; *T. bicornis* with *T. buffeli*, and *T. buffeli* with *B. caballi* [$P < 0.0004$, permutation test constrained to locations (Raup and Crick, 1979, modified by Clua et al., 2010)] (Supplementary Table S2).

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.ttbdis.2013.10.003.

Discussion

In this study, 13 tick species collected from wild and domestic ruminants were analysed by RLB hybridisation using probes allowing identification of tick-borne pathogens at the genus and species level. The majority ($n = 229$) of the 397 infections were identified at species level. Among those, 46 infections assigned to 7 species were identified by sequencing: *Ehrlichia* sp. (EU191229.1), *E. ovina*, *Theileria* sp. (giraffe), *A. platys*, *A. ovis*, *A. marginale*, and *A. bovis*. No species-specific probes were included in the assay for the former 4 species. The latter 3 species did not react with their specific oligonucleotide probes. For *A. ovis* and *A. marginale*, this was probably due to the forward position of the probes on the amplified sequence, while for *A. bovis*, it was probably due to one base pair difference in the amplified sequences. The remaining 168 infections were only identified at the genus level, even after sequencing, probably because of the presence of multiple pathogens (Microsynth, pers. communication) for which no probes were available.

Table 1
Host infestation ($n = 128$) by 13 tick species in six localities in South Africa.

Tick (**bold**) and host species	Hosts	Number of ticks				
	No. infested/examined	Larvae	Nymphs	Males	Females	Total
Rhipicephalus (B.) decoloratus						
Common eland (*Tragelaphus oryx*)	1/9 (11%)	9	91	135	97	332
Greater kudu (*T. strepsiceros*)	3/8 (38%)	1	59	35	35	130
Impala (*Aepyceros melampus*)	2/7 (29%)	213	483	434	207	1337
Southern giraffe (*Giraffa camelopardalis*)	5/5 (100%)	0	0	6	23	29
Blue wildebeest (*Connochaetes taurinus*)	4/5 (80%)	8	10	40	53	111
Sable antelope (*Hippotragus niger*)	2/2 (100%)	0	14	37	45	96
Gemsbok (*Oryx gazella gazella*)	1/1 (100%)	33	52	72	56	213
Cattle (*Bos primigenius taurus*/*B. p. taurus*/*indicus*)	17/50 (34%)	0	21	136	204	361
Sheep (*Ovis aries*)	2/14 (14%)	0	0	0	2	2
Total	37/101 (37%)	264	730	895	722	2611
R. e. evertsi						
African buffalo (*Syncerus caffer*)	14/15 (93%)	0	0	330	112	442
Common eland (*T. oryx*)	9/9 (100%)	0	2	262	27	291
Greater kudu (*T. strepsiceros*)	4/8 (50%)	7	24	7	0	38
Impala (*A. melampus*)	3/7 (43%)	0	49	11	2	62
Blue wildebeest (*C. taurinus*)	5/5 (100%)	0	0	48	38	86
Springbok (*Antidorcas marsupialis*)	4/6 (67%)	0	0	6	5	11
Southern giraffe (*G. camelopardalis*)	4/5 (80%)	0	0	15	12	27
Blesbok (*Damaliscus pygarus phillipsi*)	1/3 (33%)	3	5	0	0	8
Black wildebeest (*C. gnou*)	2/3 (67%)	0	0	3	3	6
Sable antelope (*H. niger*)	2/2 (100%)	0	0	9	1	10
Gemsbok (*O. g. gazella*)	1/1 (100%)	0	0	26	5	31
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	49/50 (98%)	189	424	431	153	1197
Sheep (*O. aries*)	5/14 (36%)	0	0	14	2	16
Total	101/128 (79%)	199	504	1162	360	2225
Margaropus winthemi						
Common eland (*T. oryx*)	6/9 (67%)	11	505	287	234	1037
Greater kudu (*T. strepsiceros*)	1/8 (13%)	0	0	0	9	9
Gemsbok (*O. g. gazella*)	1/1 (100%)	1	0	0	27	28
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	3/50 (6%)	0	0	0	11	11
Total	11/68 (16%)	12	505	287	281	1085
Amblyomma hebraeum						
Southern giraffe (*G. camelopardalis*)	5/5 (100%)	0	0	307	117	424
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	10/50 (20%)	0	0	229	114	343
Total	15/55 (27%)	0	0	536	231	767
Hyalomma m. rufipes						
Buffalo (*S. caffer*)	8/15 (53%)	0	0	13	8	21
Common eland (*T. oryx*)	9/9 (100%)	0	0	215	14	229
Southern giraffe (*G. camelopardalis*)	4/5 (80%)	0	0	19	5	24
Blue wildebeest (*C. taurinus*)	3/5 (60%)	0	0	4	7	11
Sable antelope (*H. niger*)	1/2 (50%)	0	0	1	0	1
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	28/50 (56%)	0	0	80	49	129
Sheep (*O. aries*)	2/14 (14%)	0	0	1	1	2
Total	55/100 (55%)	0	0	333	84	417
Ixodes rubicundus						
Common eland (*T. oryx*)	4/9 (44%)	0	0	22	32	54
Greater kudu (*T. strepsiceros*)	5/8 (63%)	0	0	16	34	50
Springbok (*A. marsupialis*)	5/6 (83%)	0	0	9	11	20
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	4/50 (8%)	0	0	7	21	28
Sheep (*O. aries*)	2/14 (14%)	0	0	1	1	2
Total	20/87 (23%)	0	0	55	99	154
R. appendiculatus						
Impala (*A. melampus*)	1/7 (14%)	0	0	4	0	4
Southern giraffe (*G. camelopardalis*)	1/5 (20%)	0	0	9	4	13
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	3/50 (6%)	0	0	19	2	21
Total	5/62 (8%)	0	0	32	6	38
R. gertrudae						
African buffalo (*S. caffer*)	1/15 (7%)	0	0	21	1	22
Common eland (*T. oryx*)	1/9 (11%)	0	0	8	0	8
Total	2/24 (8%)	0	0	29	1	30
R. warburtoni						
African Buffalo (*S. caffer*)	1/15 (7%)	0	0	1	0	1
Common eland (*T. oryx*)	2/9 (22%)	0	0	13	1	14
Total	3/24 (12%)	0	0	14	1	15
Haemaphysalis silacea						
African buffalo (*S. caffer*)	2/15 (13%)	0	0	0	8	8
Total	2/15 (13%)	0	0	0	8	8
R. (B.) microplus						
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	1/50 (2%)	0	3	1	3	7
Otobius megnini						
Cattle (*B. p. taurus*/*B. p. t.*/*indicus*)	2/50 (4%)	0	5	0	0	5
Total	2/50 (4%)	0	5	0	0	5
R. zambeziensis						
Impala (*A. melampus*)	1/7 (14%)	0	0	2	0	2
Total	–	475	1747	3346	1796	7364

Table 2
Infection of 8 out of 13 tick species by 23 pathogen species collected from 64 wild and 64 domestic ruminants in South Africa.

Pathogens/ticks	*R. (B.) decoloratus*	*R. e. evertsi*	*A. hebraeum*	*H. m. rufipes*	*R. appendiculatus*	*R. gertrudae*	*R. (B.) microplus*	*R. warburtoni*	*I. rubicundus*	*M. winthemi*	*H. silacea*	*O. megnini*	*R. zambeziensis*	% of pathogen species[D]
B/T genus[g]	24	30	6	6	3	0	0	0	0	0	0	0	0	69 (17.4%)
B. occultans	0	0	0	2[a,f]	0	0	0	0	0	0	0	0	0	2 (0.5%)
B. bigemina	5[b,c,e,f]	1[f]	0	0	0	0	0	0	0	0	0	0	0	6 (1.5%)
B. caballi	**7**[b,c,e]	5[b,c]	0	0	0	0	0	0	0	0	0	0	0	12 (3%)
B. sp. Sable	0	0	0	**1**[b]	0	0	0	0	0	0	0	0	0	1 (0.3%)
T. spp.	15	23	13	2	3	0	0	0	0	0	0	0	0	56 (14.1%)
T. bicornis	0	**26**[a,b,c,e]	0	0	**1**[e]	0	0	0	0	0	0	0	0	27 (6.8%)
T. ovis	2[e]	2[a,c]	0	0	0	0	0	0	0	0	0	0	0	4 (1%)
T. equi	0	14[a,b,c,e,f]	0	0	0	0	0	0	0	0	0	0	0	14 (3.5%)
T. separata	0	18[b,c,d,e]	0	0	0	0	0	0	0	0	0	0	0	18 (4.5%)
T. sp. (sable)	**9**[c,e,f]	24[b,c,d,e]	**5**[e,f]	0	0	0	0	0	0	0	0	0	0	38 (9.6%)
T. buffeli	**17**[b,c,e,f]	**17**[b,c,e,f]	0	**2**[a,f]	**1**[e]	0	0	0	0	0	0	0	0	37 (9.3%)
T. taurotragi	0	1[f]	0	**1**[b]	2[e]	0	0	0	0	0	0	0	0	4 (1%)
T. annulata	4[b,e]	2[b,e]	0	0	0	0	0	0	0	0	0	0	0	6 (1.5%)
T. sp. (kudu)	0	0	0	0	**1**[e]	0	0	0	0	0	0	0	0	1 (0.3%)
T. mutans	0	**1**[f]	1[f]	0	0	0	0	0	0	0	0	0	0	2 (0.5%)
T. sp. (giraffe)	**1**[f]	**1**[f]	0	0	0	0	0	0	0	0	0	0	0	2 (0.5%)
A/E genus	3	7	18	11	0	0	1	3	0	0	0	0	0	43 (10.8%)
A. bovis	**1**[b]	**11**[a,b,c]	0	0	0	0	0	0	0	0	0	0	0	12 (3%)
A. centrale	0	0	0	0	0	**1**[a]	0	0	0	0	0	0	0	1 (0.3%)
A. marginale	1[d]	1[c]	0	0	0	**4**[a]	0	0	0	0	0	0	0	6 (1.5%)
A. ovis	0	3[c,f]	0	0	0	0	0	0	0	0	0	0	0	3 (0.8%)
E. sp. (EU191229.1)	8[b,e]	3[c,f]	0	12[a,c]	0	0	0	0	0	0	0	0	0	23 (5.8%)
E. ovina	**1**[c]	2[a,c]	0	**1**[a]	0	**1**[a]	0	0	0	0	0	0	0	5 (1.3%)
E. ruminantium	0	0	4[f]	0	0	0	0	0	0	0	0	0	0	4 (1%)
A. platys	0	**1**[a]	0	0	0	0	0	0	0	0	0	0	0	1 (0.3%)
Prevalence of infection[A]	84/395 (21.3%)	147/909 (16.2%)	42/233 (18%)	37/259 (14.3%)	9/26 (34.6%)	6/18 (33.3%)	1/4 (25%)	3/14 (21.4%)	0/131	0/116	0/7	0/4	0/1	329/2117 (15.5%)
Pathogen mean density[B]	98/395 (0.25)	193/909 (0.21)	47/233 (0.20)	38/259 (0.15)	11/26 (0.42)	6/18 (0.33)	1/4 (0.25)	3/14 (0.21)	0/131	0/116	0/7	0/4	0/1	397/2117 (0.19)
Intensity of infection[C]	98/84 (1.17)	193/147 (1.31)	47/42 (1.12)	38/37 (1.03)	11/9 (1.22)	6/6 (1)	1/1 (1)	3/3 (1)	0	0	0	0	0	397/329 (1.21)

[A] No. of infected ticks (data not shown)/no. of tested ticks.
[B] No. of infections/no. of tested ticks.
[C] No. of infections/no. of infected ticks.
[D] % of the given pathogen species among the 397 infections.
Locations of the infections identified at species level: a, Tüssen-Die-Riviere; b, Willem Pretorius; c, Sandveld; d, Bethal; e, Thabazimbi; f, Lephalale.
In **bold:** new vector–pathogen combinations.
[g] *B/T*: includes infections reacting with this probe only; considered as belonging to the genus *Babesia*.

Table 3
Pathogen species identified from tick salivary glands by sequencing.

Identified sequence	Homology with	% of homology	Species name	Identified from
KF414714	EU191229.1	100%	Unnamed (*E.* sp.)	*H. m. rufipes* (*n* = 12)
KF414714	EU191229.1	100%	Unnamed (*E.* sp.)	*R. (B.) decoloratus* (*n* = 8)
KF414714	EU191229.1	100%	Unnamed (*E.* sp.)	*R. e. evertsi* (*n* = 3)
KF414715	AF318946.1	99%	*E. ovina*	*R. e. evertsi* (*n* = 2)
KF414715	AF318946.1	99%	*E. ovina*	*H.m.rufipes* (*n* = 1)
KF414715	AF318946.1	99%	*E. ovina*	*R. gertrudae* (*n* = 1)
KF414715	AF318946.1	99%	*E. ovina*	*R. (B.) decoloratus* (*n* = 1)
KF414716	FJ155997.1	100%	*A. bovis*	*R. e. evertsi* (*n* = 11)
KF414717	EU191231.1	100%	*A. ovis*	*R. e. evertsi* (*n* = 3)
KF414718	FJ155998.1	99%	*A. marginale*	*R. gertrudae* (*n* = 1)
KF414719	AY040853.1	99%	*A. platys*	*R. e. evertsi* (*n* = 1)
KF414720	FJ213583.1	100%	*T.* sp. (giraffe)	*R. e. evertsi* (*n* = 1)
KF414720	FJ213583.1	100%	*T.* sp. (giraffe)	*R. (B.) decoloratus* (*n* = 1)

The geographic distribution of all tick and pathogen species was congruent with their known geographic distribution except for *Hae. silacea*, *T. annulata*, and *Ehrlichia* sp. (EU191229.1). The tick *Hae. silacea* was collected at Willem Pretorius game reserve in the centre of the Free State. Its known geographic distribution is the north-eastern KwaZulu-Natal and the Eastern Cape Province (Norval, 1975; Walker, 1991; Horak et al., 1991, 2007). In Africa, *T. annulata* occurs only in northern Africa (Pipano, 1994; Jacquiet et al., 1990). Here, *T. annulata* was detected in the salivary glands of 4 *R. (B.) decoloratus* and 2 *R. e. evertsi* at Thabazimbi and in the Free State (Willem Pretorius) where they were sharing the same individual hosts. All *T. annulata* infections were identified through hybridisation with RLB-probes designed by Georges et al. (2001). Cross reactivity with pathogens identified in this study can be excluded. Whether cross reactivity occurred with an alternative or unknown *Theileria* species remains a possibility. Unfortunately, sequencing of these samples was not successful. Thus, the finding of this economically important cattle parasite in South Africa must be considered with caution. This pathogen is not discussed further, and *Ehrlichia* sp. (EU191229.1) will be discussed elsewhere (Berggoetz et al., unpublished).

Among the observed tick-pathogen combinations, 17 were congruent with the literature. Among those, 10 were recorded in the salivary glands of *R. e. evertsi*: *B. bigemina* (Büscher, 1988), *B. caballi* (De Waal and van Heerden, 1994), *T. ovis* (Jansen and Neitz, 1956), *T. equi* (De Waal and Potgieter, 1987), *T. separata* (Jansen and Neitz, 1956), *Theileria* sp. (sable) (Steyl et al., 2012), *T. taurotragi* (Lawrence et al., 1994a), *A. marginale* (Potgieter, 1981), *A. ovis* (Kaufmann, 1996), and *E. ovina* (Neitz, 1956). The remaining 7 known vector–pathogen combinations were recorded in the salivary glands of 4 tick species: *B. bigemina* (Potgieter and Els, 1977), *T. ovis* (Jansen and Neitz, 1956), and *A. marginale* (Potgieter, 1981) in *R. (B.) decoloratus*; *T. mutans* (Lawrence et al., 1994a) and *E. ruminantium* (Bezuidenhout et al., 1994) in *A. hebraeum*; *B. occultans*

Table 4a
Pathogens identified in seven tick species collected from 64 wild ruminants.

Pathogens/ticks	*R. (B.) decoloratus*	*R. e. evertsi*	*A. hebraeum*	*H.m.rufipes*	*R. appendiculatus*	*R. gertrudae*	*R. warburtoni*	% of pathogen species[d]
B/T genus[e]	16	21	2	1	3	0	0	43 (15.1%)
B. occultans	0	0	0	1	0	0	0	1 (0.4%)
B. bigemina	2	0	0	0	0	0	0	2 (0.7%)
B. caballi	6	5	0	0	0	0	0	11 (3.9%)
T. spp.	14	19	9	1	3	0	0	46 (16.1%)
T. bicornis	0	20	0	0	1	0	0	21 (7.4%)
T. ovis	2	0	0	0	0	0	0	2 (0.7%)
T. equi	0	14	0	0	0	0	0	14 (4.9%)
T. separata	0	14	0	0	0	0	0	14 (4.9%)
T. sp. (sable)	9	23	4	0	0	0	0	36 (12.6%)
T. buffeli	14	14	0	1	0	0	0	29 (10.2%)
T. taurotragi	0	1	0	1	0	0	0	2 (0.7%)
T. annulata	3	1	0	0	0	0	0	4 (1.4%)
T. sp. (giraffe)	1	1	0	0	0	0	0	2 (0.7%)
A/E genus	2	7	9	5	0	0	3	26 (9.1%)
A. bovis	0	8	0	0	0	0	0	8 (2.8%)
A. centrale	0	0	0	0	0	1	0	1 (0.4%)
A. marginale	0	0	0	0	0	4	0	4 (1.4%)
E. sp. (EU191229.1)	2	1	0	11	0	0	0	14 (4.9%)
E. ovina	1	0	0	0	0	1	0	2 (0.7%)
E. ruminantium	0	0	2	0	0	0	0	2 (0.7%)
Prevalence of infection[a]	61/235 (26%)	108/474 (22.78%)	22/91 (24.18%)	20/129 (15.5%)	7/12 (58.33%)	6/18 (33.33%)	3/14 (21.43%)	227/973 (23.33%)
Pathogen mean density[b]	72/235 (0.31)	149/474 (0.31)	26/91 (0.29)	21/129 (0.16)	7/12 (0.58)	6/18 (0.33)	3/14 (0.21)	284/973 (0.29)
Intensity of infection[c]	72/61 (1.18)	149/108 (1.38)	26/22 (1.18)	21/20 (1.05)	7/7(1)	6/6 (1)	3/3(1)	284/227 (1.25)

[a] No. of infected ticks (data not shown)/no. of tested ticks.
[b] No. of infections/no. of tested ticks.
[c] No. of infections/no. of infected ticks.
[d] % of the given pathogen species among the 284 infections.
[e] *B/T*: includes infections reacting with this probe only; considered as belonging to the genus *Babesia*.

Table 4b
Pathogens identified in six tick species collected from 64 domestic ruminants.

Pathogens/ticks	*R. (B.) decoloratus*	*R. e. evertsi*	*A. hebraeum*	*H. m. rufipes*	*R. appendiculatus*	*R. (B.) microplus*	% of pathogen species[d]
B/T genus[e]	8	9	4	5	0	0	26 (21.8%)
B. occultans	0	0	0	1	0	0	1 (0.8%)
B. bigemina	3	1	0	0	0	0	4 (3.4%)
B. caballi	1	0	0	0	0	0	1 (0.8%)
B. sp. (sable)	0	0	0	1	0	0	1 (0.8%)
T. spp.	1	4	4	1	0	0	10 (8.4%)
T. bicornis	0	6	0	0	0	0	6 (5%)
T. ovis	0	2	0	0	0	0	2 (1.7%)
T. separata	0	4	0	0	0	0	4 (3.4%)
T. sp. (sable)	0	1	1	0	0	0	2 (1.7%)
T. buffeli	3	3	0	1	1	0	8 (6.7%)
T. taurotragi	0	0	0	0	2	0	2 (1.7%)
T. annulata	1	1	0	0	0	0	2 (1.7%)
T. sp. (kudu)	0	0	0	0	1	0	1 (0.8%)
T. mutans	0	1	1	0	0	0	2 (1.7%)
A/E genus	1	0	9	6	0	1	17 (14.3%)
A. bovis	1	3	0	0	0	0	4 (3.4%)
A. marginale	1	1	0	0	0	0	2 (1.7%)
A. ovis	0	3	0	0	0	0	3 (2.5%)
E. sp. (EU191229.1)	6	2	0	1	0	0	9 (7.6%)
E. ovina	0	2	0	1	0	0	3 (2.5%)
E. ruminantium	0	0	2	0	0	0	2 (1.7%)
A. platys	0	1	0	0	0	0	1 (0.8%)
Prevalence of infection[a]	23/160 (14.4%)	39/435 (9%)	20/142 (14.1%)	17/130 (13.1%)	2/14 (14.3%)	1/4 (25%)	102/885 (11.5%)
Pathogen mean density[b]	26/160 (0.16)	44/435 (0.10)	21/142 (0.15)	17/130 (0.13)	4/14 (0.29)	1/4 (0.25)	113/885 (0.13)
Intensity of infection[c]	26/23 (1.13)	44/39 (1.13)	21/20 (1.05)	17/17 (1)	4/2 (2)	1/1 (1)	113/102 (1.11)

[a] No. of infected ticks (data not shown)/no. of tested ticks.
[b] No. of infections/no. of tested ticks.
[c] No. of infections/no. of infected ticks.
[d] % of the given pathogen species among the 113 infections.
[e] *B/T*: includes infections reacting with this probe only; considered as belonging to the genus *Babesia*.

(Thomas and Mason, 1981) and *T. taurotragi* (Lawrence et al., 1994b) in *Hy. rufipes* and *R. appendiculatus*, respectively. *Rhipicephalus e. evertsi* appears as a very important vector for *T. separata*, *T. equi*, and *Theileria* sp. (sable) in South Africa. Interestingly, *B. bigemina* was detected in the salivary glands of one *R. e. evertsi* adult. The exact role of *R. e. evertsi* in the epidemiology of redwater remains unclear (De Vos and Potgieter, 1994). According to Büscher (1988), only nymphs transmit *B. bigemina*, and transovarial transmission does not occur. Further studies are needed to evaluate the role of *R. e. evertsi* adults in the circulation of *B. bigemina*.

To our knowledge, the remaining 23 vector–pathogen combinations were never described before. Among those, 11 involved combinations with multiple records at different sites. Three vector–pathogen combinations involved a tick species, *R. gertrudae*, that was never reported as a vector (Walker et al., 2000). One third of *R. gertrudae* specimens carried bacterial pathogens, all identified at species level. Four out of 6 *A. marginale* infections were detected in this tick species suggesting a possible vector role of *R. gertrudae* in the epidemiology of gallsickness. In addition, *E. ovina* and *A. centrale* were each identified in one *R. gertrudae*. One vector–pathogen combination involved *T. bicornis* detected for the first time in ticks and previously reported in rhinoceroses (Nijhof et al., 2003; Govender et al., 2011), cattle (Muhanguzi et al., 2010), and nyala (Pfitzer et al., 2011). It was identified in the salivary glands of 26 *R. e. evertsi* at 4 localities in the lowveld and in the highveld. This strongly suggests that *R. e. evertsi* transmits *T. bicornis* on a large area. Three vector–pathogen combinations involved *T. buffeli* which has recognized vectors in some parts of the world (Gubbels et al., 2000), but to our knowledge, no identified vector in South Africa. *Theileria buffeli* infections ($n = 34$) were detected in *R. e. evertsi* and *R. (B.) decoloratus*, as well as in 2 *Hy. rufipes* at sites situated in the lowveld and highveld. This suggests that *R. e. evertsi* and *R. (B.) decoloratus*

Table 5
Within-localities comparison of tick infection prevalences and tick infection mean densities considering the four main infected tick species [*R. (B.) decoloratus*, *R. e. evertsi*, *A. hebraeum*, and *Hy. rufipes*].

Location	Tick infection prevalence			Tick pathogen mean density		
	No. infected/tested	*z*-Values	*P*-Values	Mean infection/tick	*z*-Values	*P*-Values
Sandveld (w)	77/343 (22.45%)	4.02	<0.0001	110/343 (0.32)	5.09	<0.0001
Sandveld (d)	24/251 (9.56%)			25/251 (0.10)		
Willem Pretorius (w)	14/137 (10.22%)	0.1	0.91	18/137 (0.13)	0.28	0.78
Willem Pretorius (d)	25/254 (9.84%)			29/254 (0.11)		
Tüssen-Die-Riviere (w)	32/183 (17.49%)	-0.55	0.58	33/183 (0.18)	-0.09	0.93
Tüssen-Die-Riviere (d)	21/108 (19.44%)			20/108 (0.19)		
Bethal (w)	2/3 (66.67%)	1.63	0.1	2/3 (0.67)	2.41	0.016
Bethal (d)	3/62 (4.84%)			4/62 (0.06)		
Thabazimbi (w)	41/78 (52.56%)	4.51	<0.0001	50/78 (0.64)	4.01	<0.0001
Thabazimbi (d)	10/80 (12.50%)			14/80 (0.18)		
Lephalale (w)	59/228 (25.88%)	2.43	0.01	71/228 (0.31)	2.54	0.01
Lephalale (d)	21/131 (16.3%)			21/131 (0.16)		

P-Values are considered significant when below or equal to 0.05.

play a role in the maintenance of this parasite in South Africa. Three vector–pathogen combinations involved *A. bovis* and *Theileria* sp. (sable) identified in the salivary glands of tick species that were suspected vectors. *Anaplasma bovis* was reported by Tonetti et al. (2009) in entire *R. e. evertsi* ticks. Here, *A. bovis* was detected in the salivary glands of 11 *R. e. evertsi* collected at 3 localities. Our observation corroborates previous reports and supports that *R. e. evertsi* may act as a vector for *A. bovis*. The detection of *Theileria* sp. (sable) in the salivary glands of 9 *R. (B.) decoloratus* at 3 sites and of 5 *A. hebraeum* at 2 of these sites indicates that these ticks may act as vectors in addition to *R. e. evertsi* and *R. appendiculatus* (Steyl et al., 2012). The observation of *Theileria* sp. (sable) in these 2 additional ticks contributes to explain the broad host range of *Theileria* sp. (sable) (Spitalska et al., 2005; Yusufmia et al., 2010; Pfitzer et al., 2011; Steyl et al., 2012). *Babesia caballi* has a known vector in South Africa, but it was observed in 2 new vector–pathogen combinations, involving a tick species known to transmit other pathogens. We detected *B. caballi* in the salivary glands of 7 *R. (B.) decoloratus* at 3 locations suggesting that this tick may be an additional vector of *R. e. evertsi* (De Waal et al., 1988).

The remaining 12 vector–pathogen combinations involved only one or 2 records. Among those, 5 were previously suspected. This was the case for *T. mutans* in *R. e. evertsi* (De Vos and Roos, 1981), *E. ovina* in *R. (B.) decoloratus* (Schulz, 1940) and in *Hy. rufipes* (*Hyalomma* spp., Schulz, 1940), and *T. taurotragi* in *H. rufipes* (Binta et al., 1998). In addition, *Babesia* sp. (sable) (Oosthuizen et al., 2008) that was identified in the salivary glands of *Hy. rufipes* is genetically very close to *B. occultans* (Oosthuizen et al., 2008) which is transmitted by *Hy. rufipes* (Thomas and Mason, 1981). Thus, our observation is not so surprising. The remaining 7 vector–pathogen combinations were unknown. *Theileria bicornis* was detected in one *R. appendiculatus*. *Anaplasma bovis* was detected in one *R. (B.) decoloratus*; its vector in South Africa is *R. appendiculatus* (Scott, 1994). *Anaplasma platys* which is generally associated with canine hosts (Huang et al., 2005) and *R. sanguineus* (Hoskins et al., 1991) was detected in one *R. e. evertsi*. *Theileria* sp. (giraffe) which was only known in giraffe (Oosthuizen et al., 2009) was detected in one *R. e. evertsi* and one *R. (B) decoloratus*. Finally, *Theileria* sp. (kudu), a pathogen of greater kudu (Nijhof et al., 2005) and nyala (*T. angassii*) (Pfitzer et al., 2011), and *T. buffeli* were identified in the salivary glands of *R. appendiculatus*. These 12 vector–pathogen combinations need further records for confirmation of the vector role of these ticks.

More than 70% of the collected tick species were attached to wild ruminants. Differences in infestation between wild and domestic hosts can be explained by a lower exposure to ticks of domestic animals that were treated with acaricides on a regular basis. Furthermore, domestic animals graze in managed camps restricted to several hectares while wild animal habitats are much larger and non-managed. Salivary glands of ticks that were attached to wild ruminants displayed significantly higher infection prevalences and pathogen mean densities than salivary glands of ticks attached to domestic animals at 3 localities. The pathogens that mainly contributed to the significant difference between wild and domestic animals were theilerial species transmitted only transstadially by the two-host tick *R. e. evertsi*. *Rhipicephalus e. evertsi* populations in reserves have a greater variety of hosts than those living on farms. This allows them to accumulate pathogen species originating from various hosts. This could be one explanation for the differences between salivary gland infections of ticks associated with wild vs. domestic ruminant environments. Another one could be that acaricide treatments are applied on domestic animals. This could affect the tick population structure (e.g. proportions between life stages) and therefore reduce vertical transmission and pathogen load in tick populations. Finally, differences in the susceptibility to acaricides of infected ticks and non-infected ticks could reduce the proportion of infected ticks in the environment. The frequence of given pathogen species in wild or domestic ruminants is also linked to the host preference of their vector ticks. For example, *B. bovis* transmitted by *R. (B.) microplus* in South Africa (Tonnesen et al., 2004) is almost exclusively found on cattle due to the fact that they represent the only effective hosts for *R. (B.) microplus* (Walker et al., 2003).

Here, 72% of the coinfections were observed in the two-host tick *R. e. evertsi* and only 22.6% in the one-host tick *R. (B.) decoloratus*, despite the fact that the one-host tick displayed a slightly higher infection prevalence and pathogen mean density. Tick life cycles with 2 and 3 hosts increase the chance of coinfections. Among the 529 (23×23) possibilities of coinfections, 6 pathogen associations involving 4 *Theileria* species and one *Babesia* species were significant. If coinfections in *Ixodes* ticks involving *Borrelia* spp., *Babesia* spp., *Anaplasma* spp., and *Rickettsia* spp. were described in North America (for example, Swanson et al., 2006) and Europe (for example, Lommano et al., 2012), much less is known on coinfections in ticks in southern Africa. Here, significant associations involved the most frequent pathogen species. This could indicate that associations are linked to pathogen frequency. However, the most frequent associations [*Theileria* sp. (sable) with *T. separata*, *Theileria* sp. (sable) with *T. bicornis*, and *T. separata* with *T. bicornis*] were the same as those observed in the blood of hosts exposed to these ticks (Berggoetz et al., 2013). This suggests that the associated species take advantage of their reciprocal presence. Future studies will have to understand the mechanisms of coinfections in ticks and evaluate their impact on wild and domestic ruminants.

The present study shows that many examined tick species were associated with a much broader pathogen range than previously known. *Rhipicephalus e. evertsi* and *R. (B.) decoloratus* are mainly concerned and appear as important vectors in South Africa. Pathogen species like *T. bicornis*, *Theileria* sp. (giraffe), *Theileria* sp. (kudu), and *Babesia* sp. (sable) were detected for the first time in ticks, more precisely in their salivary glands, which suggests their vector role. Furthermore, our observations showed that ticks from wild animal environments generally harboured more infections than ticks from domestic animal environments. This indicates that wild ruminants are more exposed to tick-borne pathogens which can probably be explained by the fact that they live in an uncontrolled environment with a higher host species richness than in the managed camp of domestic ruminants. Our results also shed light on the high frequency of coinfections in ticks.

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